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Computer-assisted morphometric analysis of absorptive and follicle-associated epithelia of Peyer's patches in sheep foetuses and lambs indicates the presence of distinct T- and B-cell components

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SUMMARY

The phenotypes of lymphocytes infiltrating the epithelium of the jejunal and ileal Peyer's patches in foetal sheep at about 130 days gestation and 2-month-old lambs were examined using indirect immunoperoxidase histochemistry, a panel of monoclonal antibodies and enzyme histochemistry. Computer-assisted morphometric analysis enabled the relative size of reactive areas within epithelia to be estimated. The comparison of the intraepithelial lymphocyte populations associated with structurally developed Peyer's patches of foetal sheep and those of lambs allowed assessment of the impact of extrinsic factors from which the sheep foetus is shielded. The study confirmed the postnatal expansion in the villous intraepithelial lymphocyte population and showed that this expansion involved the CD8 and $\gamma\delta$ phenotypes. CD4 lymphocytes did not appear in the follicle-associated epithelium until after birth. Unlike the villous epithelium, the follicle-associated epithelium had a high frequency of IgM^+ and MHC II^+ cells, which was dramatically reduced after birth. This postnatal reduction was particularly prominant in the follicle-associated epithelium of the jejunal Peyer's patch, where the frequency of IgM^+ cells fell from 12·4% in foetal sheep to 0·7% in lambs. Double staining for alkaline phosphatase in the jejunal Peyer's patch suggested that clusters of IgM^+ cells were associated with M cells.

INTRODUCTION

A T-cell presence in the intestinal absorptive epithelium has been well recognized. This mucosal microenvironment harbours lymphocyte subpopulations which are mostly of the cytotoxic/suppressor phenotype ($CD8^+$),[1] although a smaller population of lymphocytes without T- or B-cell markers is present in mice and rats.[2,3] Intraepithelial lymphocytes (IEL) are thought to be important in cell-mediated intestinal immune responses, in the regulation of secretory immunity and in the mediation of systemic tolerance.[4,5] Lymphocytes with $\gamma\delta$ T-cell receptor have recently been shown to have a predilection for the intestinal epithelium,[6] but little is known of their function. Possible roles include surveillance of surface molecules in the intestinal epithelium and elimination of altered cells.[7] It has been shown that lumenal antigens influence the number of IEL. Thus, germ-free mice show low numbers of IEL,[8,9] while numbers of IEL are increased in some intestinal diseases such as coeliac disease[10] and intestinal protozoal infections.[11] In lambs the marked increase in the number of IEL that occurs after birth has been related to postnatal confrontation with antigen.[12]

In contrast to the absorptive epithelium, the follicle-associated epithelium (FAE) over the Peyer's patches (PP) has been reported to show a high frequency of T cells with the CD4 phenotype, and also B cells.[3,13,14] The FAE over the PP is thought to have a special role in the transport of antigen from the intestinal lumen to the follicle, a function consigned to the M cells.[15] The presence of B cells and $CD4^+$ cells in the FAE may suggest involvement in an immune response against transported antigen.

The sheep foetus represents an opportunity to examine immune cell populations developing independently of external antigen. The sheep placenta is impermeable even to small molecules[16] and there is no transfer of maternal immunoglobulins to the sheep foetus.[17] However, despite the absence of external antigen, lymphoid organs such as PP and lymph nodes are well developed before birth. M cells have been observed to envelop lymphocytes in the jejunal PP of sheep foetuses[18] but the

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Abbreviations: BSA, bovine serum albumin; CD, cluster of differentiation; FAE, follicle-associated epithelium; IEL, intraepithelial lymphocyte(s); Ig, immunoglobulin; mAb, monoclonal antibody; PBS, phosphate-buffered saline; PP, Peyer's patch(es); VE, absorptive (villous) epithelium.

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Table 1. Monoclonal antibodies to sheep leucocytes surface molecules used in this study

Surface molecules	Antibodies	Cells marked/function	Reference
CD4	ST4*	T-helper cells	26
CD5	ST1*	T cells, some B cells	27
CD8	ST8*	T cytotoxic cells	28
IgM	Plg 45A	B cells	From VMRD Inc., Pullman, WA
IgM	McM9§	B cells	29
MHC I	SBU.I†	MHC class I antigens	30
MHC II	SW73·2‡	MHC class II antigens	31
$\gamma\delta$ TcR	86D†	$\gamma\delta$ T-cell subset	32

Monoclonal antibodies were provided by Dr W. Hein* and Dr C. Mackay,† Basel Institute for Immunology, Switzerland, Dr J. Hopkins,‡ University of Edinburgh, U.K. and Dr K. Beh,§ CSIRO, Australia.

phenotype of the enfolded lymphocytes has not been determined. In the present study the IEL populations of the FAE of structurally developed jejunal and ileal PP in foetuses were compared with PP of postnatal lambs to assess the impact of extrinsic factors. We also compared FAE lymphocyte populations with those of the adjacent villous epithelium. We confirm the previous observation[12] of a postnatal expansion in the villous IEL population and show that this expansion involves the CD8 and $\gamma\delta$ phenotypes. After birth CD4 lymphocytes appear in the FAE. Unlike the villous epithelium, the FAE has a high frequency of IgM^+ and major histocompatibility complex (MHC) II^+ cells which is dramatically reduced after birth and is much less frequent in the ileal than in the jejunal PP. Staining for alkaline phosphatase suggests that the IgM^+ lymphocytes occur in clusters in M cells.

MATERIALS AND METHODS

Animals and tissues

Sheep of the Dala breed were obtained from Heggedal research station, Asker, Norway. Five sheep foetuses aged (±1 day) 128 ($n=2$), 132 (2) and 135 (1) days of gestation and seven lambs aged 18 (1), 28 (1), 45 (1), 54 (1), 49 (2) and 90 (1) days were used. Ages (gestation 150 days in sheep) were determined from observed date of mating and time of lambing.

Tissue pieces were collected under pentobarbital anaesthesia. Sections were taken from the ileal PP at the attachment of the ileocecal fold to the ileum, and from the jejunal PP at random in the middle part of the jejunum. The tissue was frozen in monocholorodifluoromethane (Prestogas, ICI, Cheshire, U.K.) chilled with liquid nitrogen, and stored at −70°. To protect the mucosa during freezing and sectioning, the tissue pieces were placed with the mucosa down onto pieces of liver.

Antibodies

Monoclonal antibodies used in this study were peroxidase-conjugated sheep anti-mouse Ig (RPN 931; Amersham, Amersham, U.K.) and murine monoclonal antibodies against sheep lymphocyte determinants (Table 1).

Immunohistochemical staining

Sections 6 μm in thickness, cut on a cryostat and collected on microscope slides precoated with a solution containing 0·1% gelatin and 0·01% chromium potassium sulphate, were allowed to dry for 2 hr and then fixed for 10 min in acetone. Unless otherwise stated, all incubations were at room temperature and washes were for 5 min in Dulbecco's phosphate-buffered saline (PBS), pH 7·4. After fixation the sections were air dried for 5 min, briefly warmed with a hair dryer and rehydrated in PBS. To inhibit endogenous peroxidase, sections were incubated in 0·05% phenyl hydrazine (Fluka, Buchs, Switzerland) in PBS for 40 min at 37°C. After two washes in PBS, monoclonal antibody (mAb) was added to the sections and incubated for 30 min in a humid chamber, washed, and then incubated for 30 min with peroxidase-conjugated sheep anti-mouse Ig (RPN 931; Amersham) diluted 1:50 in PBS with 12·5% bovine serum albumin (BSA). Following washing in PBS, peroxidase activity in the sections was detected by incubation for 5 min in a solution of 0·05 M Tris HCl buffer, pH 7·6 containing 0·6 mg/ml diaminobenzidine hydrochloride acid (Sigma, St Louis, MO), 0·01% hydrogen peroxide (Merck, Darmstadt, Germany). The reaction was terminated by rinsing in PBS for 5 min. The reaction product was intensified by exposure to 0·1% osmium tetroxide for 30 seconds and then washed in distilled water. The sections were counterstained with 2% methyl green in distilled water for 10 min and coverslipped with Aquamount (BDH Ltd, Poole, Dorset, U.K.). Control sections were incubated with PBS and all primary antibodies were undiluted culture supernatants, except 86D which was ascites fluid diluted 1:500 in PBS with 12·5% BSA.

Alkaline phosphatase

The demonstration of M cells was based on the absence of alkaline phosphatase in the brush border of these cells.[19] Unfixed 6 μm cryostat section on gelatin slides were stained for alkaline phosphatase with naphthol phosphoric acid and fast red violet for 6 min. Briefly, 2 mg naphthol, AS-MS phosphate (N 4875; Sigma) dissolved in 0·02 ml N,N-dimethylformamide and 0·01 ml 1M levamisole (L9756; Sigma) were mixed with 9·8 ml 0·1 M Tris HCl buffer, pH 8·2, containing 10 mg fast red TR salt (F 1500; Sigma). The solution was prepared immediately before staining and filtered through ordinary filter paper directly onto the slides.

Computer-assisted morphometric analysis

The first five FAEs and regions of adjacent villous epithelia encountered when scanning immunohistochemical sections from each animal were analysed using a video-image analysis system (Zeus™, A/S, Pixelwerks Ltd, Bergen, Norway). Five

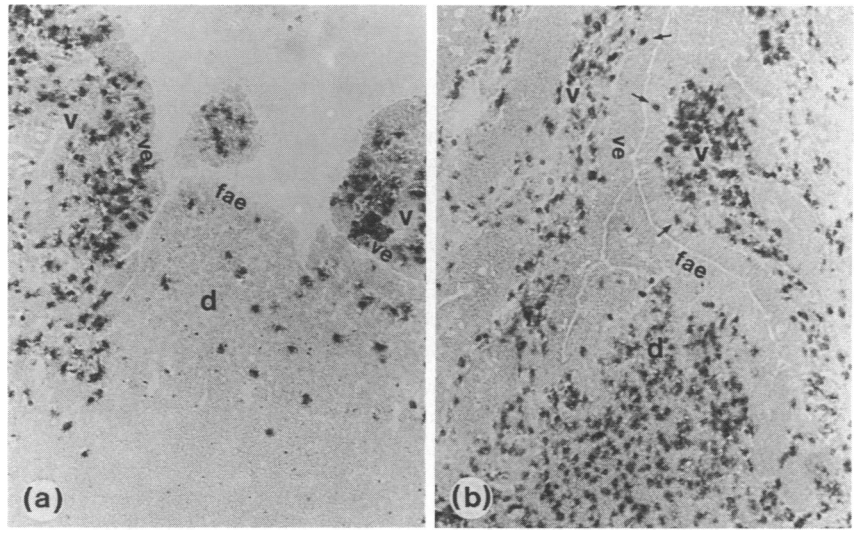

Figure 1. Jejunal PP, 2-month-old lamb. (a) $\gamma\delta$ (86D) T cells are frequent in the absorptive epithelia (ve) and lamina propria of the intestinal villi (v) but less frequent in the follicle-associated epithelium (fae) overlying the dome (d). (b) Few CD4 (ST4) T cells (arrows) are present in the absorptive epithelium along the villi. × 88.

sheep foetuses aged between 128 and 135 days of gestation and five lambs aged between 45 and 90 days were compared. The four lymphocyte subpopulations assessed included B cells (IgM^+) and CD4 (ST4), CD8 (ST8) and $\gamma\delta$ (86D) T cells.

Strips of either 18 μm or 10 μm in width were selected from the FAE and adsorptive (villous) epithelium (VE) and their area determined. The strips were drawn within the epithelium following the contours of the epithelial nuclei and luminal border and were estimated to represent up to 85% of the epithelial width. Appropriate grey level thresholds were set to distinguish stained from unstained tissue and the total stained area was determined. Regions of epithelia that were cut tangentially or close to crypts were excluded.

The percentage of the selected area of epithelia occupied by lymphocyte subpopulations was calculated. The significance of differences in means of various groups was assessed by the Student's t-test. The level of significance chosen was $P < 0{\cdot}05$.

RESULTS

The IEL present in the VE associated with the PP of foetal sheep and lambs reacted with monoclonal antibodies against T-cell surface molecules. The labelled lymphocytes were predominantly of the $CD8^+$ and $\gamma\delta^+$ (Fig. 1a) subsets, although some $CD4^+$ cells were detected (Fig. 1b).

The FAE of jejunal and ileal PP in both foetuses and lambs contained cells staining for B and T surface molecules. The presence of cells staining for the B-cell marker IgM was particularly prominent in the jejunal PP of foetuses and young lambs (< 4 weeks old). IgM^+ cells were often observed to occur in clusters in the FAE of the jejunal PP (Fig. 2). Similar clusters were less obvious in the FAE of the ileal PP and clusters were not observed with staining for the T-cell markers ST1 (CD5), ST4 (CD4), ST8 (CD8) and 86D ($\gamma\delta$). Nor was the pattern observed with staining for MHC 1 antigens. The epithelial cells of the FAE and VE did not stain for MHC II antigens (Fig. 3).

In the jejunal PP, enzyme staining for alkaline phosphatase demonstrated that IgM^+ cell clusters were frequently associated with breaks in staining, which are suggestive of M cells (Fig. 4).

Computer-assisted morphometric analysis confirmed the postnatal expansion of the IEL population in the VE associated with the PP. The percentage of IEL in the VE of both the jejunal and ileal PP increased significantly from 3·6% and 1·7%, respectively, in foetuses to 7·7% and 6·6% in lambs. There was a significant decrease in the percentage of IEL in the FAE of jejunal PP of lambs (3·2%) compared to foetuses (13·5%). A similar decrease in IEL was not observed in the FAE of the ileal PP (2·2% in foetuses to 1·9% in lambs; not significant).

Examination of lymphocyte subsets revealed that in foetuses IgM^+ cells occupied 12·4% of the area of FAE in jejunal PP (Fig. 5a), which was significantly larger than the 1·8% of area occupied in the FAE of the foetal ileal PP (Fig. 5b). In lambs, both areas decreased to about 0·7% and 0·5% in the jejunal PP and ileal PP, respectively. This was a significant change for the jejunal PP but not the ileal PP. The VE of ileal PP and jejunal PP in both foetuses and lambs contained no IgM^+ areas.

Areas staining for $CD4^+$ cells were not found in the FAE of PPs in foetuses and only about 0·01% of the foetal VE was stained with this antibody. In lambs, $CD4^+$ cells occupied less than 0·8% of the FAE and VE. The differences in areas of $CD4^+$ cells between absorptive epithelia of foetuses and lambs and between the epithelia of the PPs of lambs were not significant.

The percentage of the foetal FAEs occupied by $CD8^+$ cells was less than 0·4% and decreased further in lambs to be only about 0·1% (not significant). The areas of $CD8^+$ cells in the VE of jejunal PP and ileal PP increased from 0·6% and 0·4% in foetuses to be 1·4% and 1·6% in lambs, respectively. However, only the increase observed in the jejunal PP was significant.

A comparison of CD4:CD8 staining areas in FAE of lambs produced similar ratios in the jejunal and ileal PP, 6·01 and 6·45, but in the VE there were lower ratios, 0·45 and 0·07. The increased presence of $CD4^+$ cells in the FAE of lambs was further revealed by direct comparison with $CD4^+$ areas in the

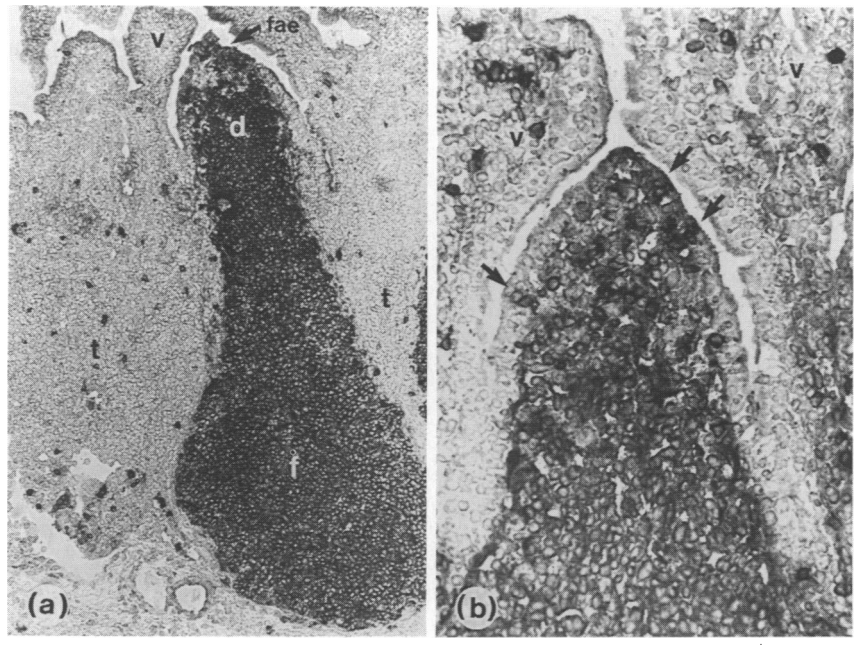

Figure 2. (a) Jejunal PP, 135 days gestation sheep foetus. IgM (McM9) cells extend from the follicle (f) through the dome (d) into the follicle-associated epithelium (fae). × 88. (b) Ileal PP, 4-week-old lamb. IgM cells appear to be present in groups of three or less (arrows) in the follicle-associated epithelium. t = T-cell area; v = villi. × 210.

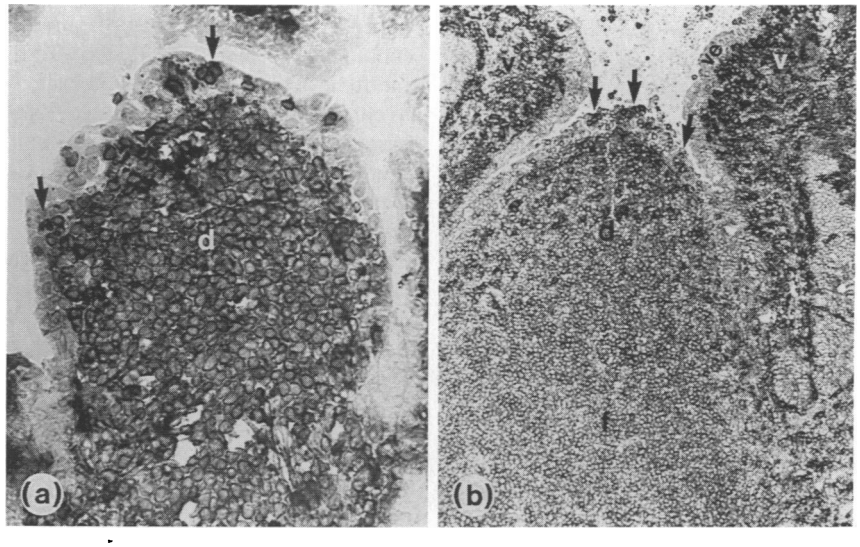

Figure 3. Jejunal PP (a) 135 days gestation sheep foetus. Groups (arrows) or single MHC II (SW73.2) cells are present in the follicle-associated epithelium overlying the dome (d). Neither absorptive nor follicle-associated epithelia show any reaction for MHC II. × 210. (b) four-week-old lamb. Larger clusters of MHC II cells (arrows) in the follicle-associated epithelium and none are present in the absorptive epithelium (ve). f = follicle; v = villi. × 88.

VE which yielded ratios of 1·22 and 4·77 in the jejunal and ileal PP.

The area of the foetal VE in the jejunal PP and ileal PP which stained for $\gamma\delta^+$cells was 3% and 1·3%, respectively. These areas were significantly larger than the areas occupied by $\gamma\delta^+$ cells in their adjacent FAE. In lambs, the area of $\gamma\delta^+$ cells in the VE increased significantly in both the jejunal PP and ileal PP to be 5·6% and 4·8%, respectively, and both areas remained significantly larger than their FAE.

DISCUSSION

The present study confirms and extends the previous observations on the postnatal expansion of IEL in sheep.[12] We have demonstrated that most IEL in the absorptive epithelium were T cells and that the expanding populations were predominantly of the $\gamma\delta$ and CD8 subsets. The absence of staining for B-cell markers was consistent with previous investigations in other species.[1] The present study has also shown that the IEL

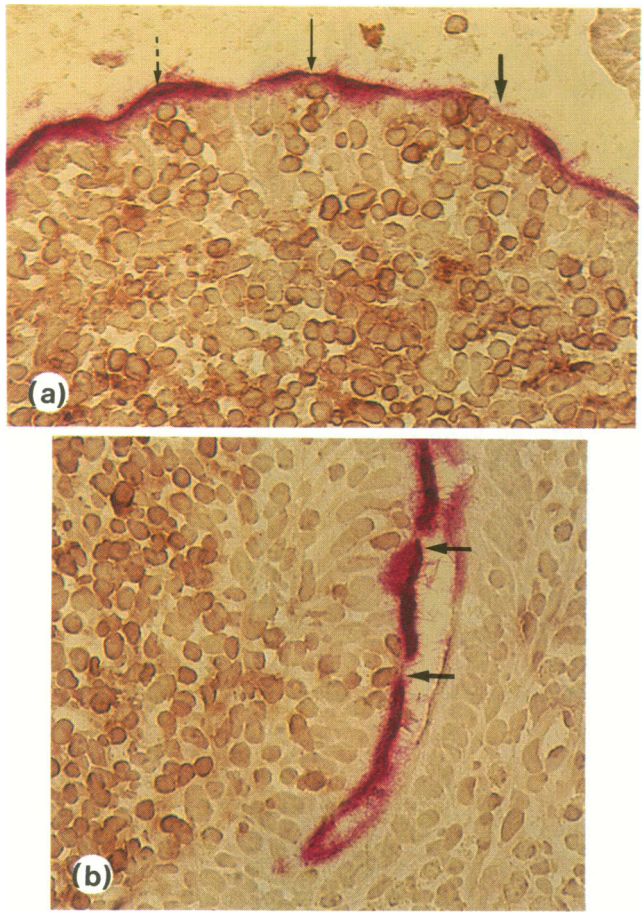

Figure 4. Jejunal PP, 4-week-old lamb. (a) At the top of the dome (d), large clusters of IgM (McM9) cells are frequently associated with interruptions (large arrow) or thinning (small arrow) of alkaline phosphatase reactivity in the luminal border of the follicle-associated epithelium but are sometimes apparently unassociated (dotted arrow) with changes in luminal border reactivity. × 245. (b) At the base of the dome, similar interruptions to luminal border reactivity are seen (arrows) which tend to associate with fewer IgM cells. × 392.

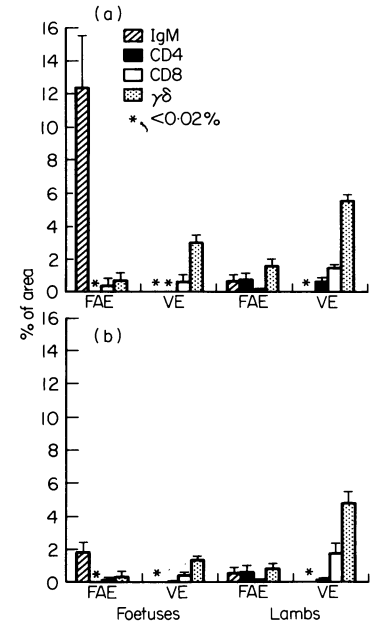

Figure 5. The percentage of area occupied by lymphocytes in the follicle-associated (FAE) and absorptive epithelia (VE) of the (a) jejunal and (b) ileal Peyer's patches of foetuses and lambs.

populating the FAE covering the domes are different to lymphocytes in the villous epithelium. The comparison of ratios of various T-cell subsets in the different epithelial compartments agreed with the observations of other investigators in that the FAE had a proportionally stronger presence of $CD4^+$ cells than the VE.[14] Indeed the CD4 presence may have been underestimated by the method of counting used in this study which was intended to exclude subepithelial lymphocytes and so may also have excluded some IEL associated with the basement membrane. $CD4^+$ IEL have been observed to associate with the basement membrane.[1]

The preference for B lymphocytes in the FAE of the foetus and in the jejunal PP was remarkable, particularly given the non-random distribution of the B cells. Staining for alkaline phosphatase, previously used to distinguish M cells in PP of mouse and man,[14,19] combined with immunoperoxidase staining for IgM suggested that clusters of B cells were associated with M cells. M cells have been so termed to describe their relation to lymphocytes: these specialized epithelial cells envelop a group of lymphocytes and form a membrane (M) between the lymphocytes and the lumen (definition: Owen, 1977).[20] The occurrence of lymphocytes adjacent to M cells has been seen in the context of an antigen-sampling function for the M cells. M cells are thought to sample antigen from the intestinal lumen for subsequent presentation in lymphoid tissue. The FAE overlies the dome of the PP, which has been shown in a number of species to contain all the components necessary for the initiation of an immune response. In rats,[21] mice[13] and sheep,[22] the dome has been found to be rich in dendritic cells, macrophages and T and B lymphocytes.

A predominance of B cells has been observed in the FAE of PP in mice , and B cells have been found in the leucocyte clusters enfolded by M cells.[13,19] In humans, Bjerke *et al.*[14] found T cells to be more numerous in the FAE than B cells and also reported T cells to be in clusters. The occurrence of $CD4^+$ lymphocytes and $MHC\ II^+$ dendritic cells adjacent to human M cells led these investigators to suggest that M cells could play an important role in the induction of immune response. Using immunoelectron microscopy, Jarry *et al.*[3] identified IgM^+ lymphocytes, some $CD8^+$ lymphocytes and a few $CD4^+$ lymphocytes in contact with rat M cells. The present study cannot exclude the possibility that M cells are infiltrated by T cells but they do not appear to form clusters in sheep.

It is tempting to consider the foetal and neonatal FAE as an extension of the dome/follicle unit forming a single B-cell milieu. A constantly renewed FAE integrated in a B-cell compartment might imply either frequent exchange of lymphocytes between the FAE and the follicle or local proliferation of B cells within the FAE. While the present study cannot rule out either possibility, other investigators have favoured migration of lymphocytes into the epithelium.[2,4] Further studies are currently being undertaken to resolve this question in sheep.

A point of interest in the sheep foetus relevent to the high frequency of B cell clusters in the FAE of the jejunal PP is the putative function of M cells. The sheep placenta does not allow transfer of immunoglobulins and excludes even small mol-

ecules.[16,17] The presence of the B cells in the FAE is therefore not dependent on postnatal phenomena, including confrontation with antigen, although a function related to antigen after birth cannot be excluded. Indeed, a novel observation of this study is the change in nature of the IEL populations between foetuses and lambs. The expanded presence of T cells in older lambs would argue for co-operation between various cell types in handling the onslaught of external antigen in the postnatal animal. However, the strength of the B-cell presence in foetuses and its subsequent decline in lambs are more difficult to explain. Further kinetic and functional data on the respective lymphocyte populations would be needed to clarify their significance.

A functional difference between the ileal and jejunal PP[23] may provide an explanation for the different B-cell distribution and lower overall B-cell presence in the FAE of the ileal PP. The ileal FAE is comprised of a uniform population of cells which resemble M cells of the jejunal PP in their capacity for macromolecular transcytosis.[18] However, in contrast to M cells, the macromolecules are included in vacuoles containing 50 nm membrane-bounded particles reactive for carbonic anhydrase (CAP).[18,25] These vacuoles subsequently empty macromolecules and CAP to the extracellular space. CAP have been observed to be taken up by lymphocytes in the follicle centre.[24,25] It is speculated that this process may represent a significant epithelial/B-cell interaction. The clustering of B cells in M cells of the jejunal PP may signify a similar need but an alternative mechanism for contact between epithelial cells and B lymphocytes.

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